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Abstract

Radiation-induced bystander signaling has been found to occur in live rainbow trout fish (*Oncorhynchus mykiss*). This article reports identification of key proteomic changes in a bystander reporter cell line (HaCaT) grown in low-dose irradiated tissue-conditioned media (ITCM) from rainbow trout fish. In vitro explant cultures were generated from the skin of fish previously exposed to low doses (0.1 and 0.5 Gy) of X-ray radiation in vivo. The ITCM was harvested from all donor explant cultures and placed on recipient HaCaT cells to observe any change in protein expression caused by the bystander signals. Proteomic methods using 2-dimensional (2D) gel electrophoresis and mass spectroscopy were employed to screen for novel proteins expressed. The proteomic changes measured in HaCaT cells receiving the ITCM revealed that exposure to 0.5 Gy induced an upregulation of annexin A2 and cingulin and a downregulation of Rho-GDI2, F-actin-capping protein subunit beta, microtubule-associated protein RP/EB family member, and I4-3-3 proteins. The 0.1 Gy dose also induced a downregulation of Rho-GDI2, hMMS19, F-actin-capping protein subunit beta, and microtubule-associated protein RP/EB family member proteins. The proteins reported may influence apoptotic signaling, as the results were suggestive of an induction of cell communication, repair mechanisms, and dysregulation of growth signals.

Keywords

ionizing radiation, bystander effects, low dose, HaCaT, proteomics, annexin A2

Introduction

Over the past 2 decades, there has been great interest and scientific effort focusing on the phenomena of nontargeted radiation effects, which have been intensively studied and reviewed (Mothersill et al. 2012), in particular radiation-induced bystander effects (RIBEs). The RIBEs do not demonstrate a linear dose–response relationship, which challenges the classical target theory (Belyakov, 2005). Previous assumptions that DNA was the target necessary for ionizing radiation (IR) to successfully damage the cell (Hall and Giaccia, 2012) are now considered to be incomplete. Early studies of RIBE employing medium-transfer techniques using human epithelial cells (C. Mothersill and Seymour, 1997) led to the discovery that a factor(s) of some sort may be involved in transmission of a bystander signal, and cell–cell contact is not always necessary for signal transmission, confirmed by experiments in which cells were not in direct contact and also by inhibiting gap-junction communication (Mothersill and Seymour, 1998). Fundamental cellular events central to the overall process of RIBE include chromosomal rearrangements, gene mutations, apoptosis, and

genomic instability (Morgan and Sowa, 2007). Experimental end points associated with RIBE include generation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and an induction of calcium (Ca^{2+}) signaling in bystander cells after exposure to radiation-induced bystander medium (Narayanan et al. 1997; Lyng et al. 2000; Azzam et al. 2012). The bystander factor is still unknown, and the specific molecular events and

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signaling entities are still not completely understood. However, recently exosomes have emerged as a possible candidate for the bystander “factor” signaling in the MCF7 (Al-Mayah et al. 2012) and HaCaT cell lines (Jella et al. 2014). Exosomes are well-known mediators of communication between cells and could be responsible for the transfer of cellular communication between irradiated and unirradiated (bystander) cells.

To date most work investigating RIBE has been carried out in vitro. Investigations have verified fish cell lines for bystander studies, including EPC (endothelial progenitor cell), RTG-2 (*Oncorhynchus mykiss* cell line; testis ovary mix), RT-Gill (*Oncorhynchus mykiss* cell line; gill) W1, and CHSE-214 (embryonic fish cell line) (O’Neill-Mehlenbacher et al. 2007). The investigation revealed 2 significant outcomes: (1) bystander signal production and cellular response can vary depending on the cell line in question and (2) production of a bystander signal and response are in fact independent processes. Evidence for bystander effects has also been discovered in fish and mice (Surinov et al. 2001), suggestive of an evolutionary conserved process in place. Mothersill and colleagues have investigated the communication of bystander signals in vivo, utilizing tissue explant techniques (Mothersill et al. 1990; Mothersill 1998; Mothersill et al. 2001). Briefly, the technique involves tissue irradiated in vivo or ex vivo, followed by the harvest and filtration of growth media, and their transfer onto either unirradiated tissue or reporter cell lines (Mothersill et al. 2001; Mothersill et al. 2005). Measurement of specific end points includes cell survival, cell death, or various biochemical parameters, allowing identification of the key cellular mechanisms. A reduction in cell survival is indicative of communicated bystander signals, as shown by cell viability experiments using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Howe et al. 2009) or clonogenic assays (Gow et al. 2008).

An extensive amount of work has determined that irradiation-induced bystander signaling can occur between both fish and mammals. One experiment in particular revealed that bystander signals can be passed from irradiated rainbow trout (*Oncorhynchus mykiss*) to neighboring nonirradiated fish, via release of a chemical component into the water surrounding the fish, indicative of bystander factor(s) (Mothersill et al. 2006). Others studies have shown that zebrafish (*Danio rerio*; Mothersill et al. 2007) are capable of producing bystander signals and have demonstrated that various sensitivity levels exist among the individual fish. Experiments involving Japanese Medaka (*Oryzias latipes*) showed that bystander signals are stronger when emitted or received by repair-deficient cells, which are understandably more sensitive to damage (Mothersill et al. 2009). Additionally, a role for serotonin in the bystander signaling response has been investigated and may be a potential contender in bystander signaling (Mothersill et al. 2010; Fazzari et al. 2012; Lyng et al. 2012), particularly in zebrafish (*Danio rerio*; Saroya et al. 2009). In the current investigation, attempts were made to bridge the in vitro versus in vivo gap on the bystander effects using a well-known bystander reporter cell line (HaCaT; Mothersill & Seymour 1997; Furlong et al.

2013). The cells were grown in irradiated tissue-conditioned media (ITCM) harvested from irradiated fish explants. Through 2D difference gel electrophoresis (2D DIGE) coupled with mass spectroscopy (MS), the changes in protein expression were reported and compared to previous proteomic studies on rainbow trout, with the aim of elucidating a clearer bystander proteomic signaling response. Demonstrating bystander effects in fish indicates the need for a more rigorous risk assessment of the risks associated with low-dose radiation and in particular bystander radiation effects.

Materials and Methods

Ethics

All fish and tissue were obtained and handled according to guidelines at McMaster University, and the procedures were covered by the Animal Utilization Protocol (AUP) 06-21-01.

Direct Irradiation, Tissue Explant Technique, and Harvest of ITCM

All rainbow trout (*O mykiss*) fish were sourced from Humber Springs Trout Farm (Orangeville, Ontario, Canada) and housed at McMaster University, Hamilton, Ontario, Canada. A portable X-ray unit (Faxitron X-ray Corporation cabinet X-ray system, Wheeling, Illinois) was used to deliver a whole body mean dose of 0.1 and 0.5 Gy to the fish and was previously calibrated using thermoluminescence dosimeters and established in 2006 (Mothersill et al. 2006). The fish weighed approximately 20 to 25 g. It was not possible to aerate the water or control temperature during irradiation time. For irradiation, the fish were placed in groups of 2 in covered containers containing 5 L water, and the irradiation process took 5 minutes. Following irradiations, the fish were placed in containers that were aerated during the entire experiment and maintained at 19°C (different containers for different doses). Handling and confinement has been investigated to ensure the well-being of the fish in our previous studies (Mothersill et al. 2009, 2007, 2006). The fish were left for a 4-hour period to allow for bystander signal accumulation within the fish, after which they were euthanized following McMaster’s Animal Research Ethics Board (AREB) and The Canadian Council on Animal Care (CCAC) guidelines. Skin epidermis was excised immediately for subsequent tissue explant culture. Unirradiated fish were used as controls, and tissue was collected following the same procedures and guidelines.

The fish tissue explants were prepared following the technique described by Mothersill and colleagues (Mothersill et al. 1988). The excised tissue was transported immediately in Rosewell Park Memorial Institute 1640 (RPMI-1640) medium (Gibco, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Burlington, Ontario, Canada), 5 mL of 200 mmol/L L-glutamine (Gibco), 0.5 µg/mL hydrocortisone (Sigma-Aldrich, Oakville, Ontario, Canada), and 12.2 mL of 1 mol/L Hepes buffer (Gibco). The

supplemented RPMI-1640 medium was used throughout the experiment. Each piece of skin was carefully poured into a Petri dish and cut into 3 smaller pieces of approximately 2 to 3 mm². Tissue culture flasks (T25; BD-Beckton Dickinson, Canada) were prepared with 2 mL RPMI and each one labelled accordingly. Flasks were stacked in an incubator at 19°C for 2 days to allow explants to attach, grow, and release bystander signals to the culture medium. Tissue culture flasks were set up as follows: 6 fish per treatment group (0 Gy, n = 6; 0.1 Gy, n = 6; and 0.5 Gy, n = 6). Multiple explants were prepared from each fish, and the subsequent media for harvest were pooled per individual fish. Tissue explants were closely monitored for 2 days.

Media from the irradiated tissue explants (ITCM) was carefully harvested after the 2 day incubation period, making sure not to disrupt intact tissue explants, and the harvested ITCM was filtered with a 0.22- μ m filter with HT Turffryn Membrane (Pall Life Sciences, US). The explants from which the media were harvested were replenished with fresh RPMI-1640, and tissue explants were reincubated at 19°C and after 10 days of incubation fixed in 10% formalin.

Exposure of HaCaT cells to ITCM for proteomic analysis. Prior to irradiation, 5×10^4 HaCaT cells were seeded into each well of a sterile 6-well plate (BD, Oakville, Ontario, Canada) covered with 3 mL of RPMI-1640 (Gibco) supplemented media mentioned previously and allowed to grow for 2 to 3 days and incubated at 37°C with 5% CO₂ in air. Three 6-well plates were set up in total for the experiment; 1 plate per corresponding dose of ITCM harvested from the irradiated fish (n = 6; plate 1 = 0 Gy, plate 2 = 0.1 Gy, and plate 3 = 0.5 Gy). Initially, the cells were grown in normal media and monitored on a daily basis and allowed to reach approximately 70% to 80% confluency. Once the HaCaT cells had reached optimal confluency, they were washed with phosphate-buffered saline (PBS; Gibco). The ITCM was then added to the cells that were reincubated at 37°C for 4 hours to allow for bystander signal transmission.

Exposure of HaCaT cells to ICCM for real-time PCR analysis. The direct irradiation exposure and harvest of the irradiated cell conditioned media (ICCM) and RNA extraction were equivalent to the procedure described in Furlong et al. (2013). Briefly, 2×10^5 of HaCaT cells were plated into T25 flasks, with 5 mL of RPMI-1640 supplemented media. A set of flasks were set up in triplicate for direct irradiation and for harvesting of bystander media for each irradiation dose (0, 0.05, and 0.5 Gy). Another set of flasks were set up as bystander recipients, also in triplicate. Cells were grown for 2 to 3 days and incubated at 37°C with 5% CO₂ in air before irradiation with a Cobalt60 radiation source. The media (ICCM) were harvested from the first set of flasks (directly irradiated) 1 hour postirradiation and pooled per triplicate flask at each of the time points (1, 4, 8, and 24 hours) and each of the dose points (0, 0.05, and 0.5 Gy). The second set of flasks was then exposed to the harvested ICCM for 1, 4, 8 or 24 hours.

Exposure of HaCaT cells to ITCM for MTT assay. HaCaT cells were seeded in 96-well microplates (Nunc, Denmark) at a density of 1×10^4 cells/well in 100 μ L Dulbecco Modified Eagle Medium (DMEM) F12 (Gibco) medium containing 10% FBS. After 24 hours of cell attachment, plates were washed with 100 μ L/well PBS, and the cells were treated with 0, 0.1, and 0.5 Gy ITCM for 24 hours. Six replicate wells were used for each control and test dose per microplate. Cell viability was assessed using MTT assay outlined subsequently.

Proteomic Experiment

Protein extraction. Following the incubation periods, ITCM was poured out of the flasks, and cells were washed in ice-cold PBS. Protein lysis buffer containing 8 mol/L urea containing 10% (v/v) 0.5 mol/L Tris-HCl (pH 7.4), 0.02 mol/L EDTA, 0.05 mol/L dithiothreitol, 10% (v/v) glycerol, 6% (v/v) ampholytes (Resolyte, pH 3.5-10; Merck-BDH, Quebec, Canada), 2% (v/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), 0.2 mg mL⁻¹ RNase, and 0.2 mg mL⁻¹ DNase (Smith et al. 2005) was used to isolate protein. Cell lysates were centrifuged at 4°C for 5 minutes at 10 000g and desalted using a commercially available kit (Thermo Scientific, Ontario, Canada) to produce a higher quantity of protein. Total protein content was quantified using the Bradford Assay (Biorad, Mississauga, Ontario, Canada), and 45 μ g was taken from each sample for subsequent 2D gel electrophoresis.

Two-dimensional electrophoresis. All electrophoresis was carried out using Protean isoelectric focusing (IEF) system (Biorad, Mississauga, Ontario, Canada), following the manufacturer's instructions and using rehydration/solubilization, equilibration, and running buffers supplied by Biorad. The quantified protein extracts from HaCaT cells were mixed with reswelling buffer. Protein mixture of 125 μ L was used to rehydrate a pH 4-7 immobilized pH gradient (IPG) strips. Each protein mixture corresponded to a dose (0 Gy n = 6, 0.1 Gy n = 6, and 0.5 Gy n = 6) and was resolved on a separate gel, yielding 18 gels in total. The IPG strips were rehydrated overnight, at room temperature, with rehydration/solubilization buffer. The IEF involved a ramped voltage change delivered over 3 steps up to a maximum of 20 000 V. After IPG strip equilibration, each strip was placed onto a 10% to 15% gradient polyacrylamide slab gel (8 \times 7 cm) for the second dimension (2D) electrophoresis. The 2D was resolved on a 1 \times Tris/glycine gel (Biorad) and proteins separated by size (molecular weight) in a direction perpendicular to the first dimension run on the Protean 2D casting and running apparatus. Twenty-five mmol/L Tris, 192 mmol/L glycine, and 0.1% sodium dodecyl sulfate (SDS) buffers were added to the upper and lower tank, respectively; maximum voltage = 200 V and running time = 45 minutes. After electrophoresis, the gels were fixed with 10% methanol, 7% acetic acid, and water, and stained with SYPRO-ruby stain followed by destaining in 10% ethanol.

Table 1. List of Forward and Reverse Oligo Sequences of *Anxa2* and Actin.

Gene	Forward Oligo Sequence	Reverse Oligo Sequence
ANXA2	5'ACAGCCATCAAGACCAAGG'3	5'CAAATCACCCTCCAGGT'3
Actin	5'ACTCTTCCAGCCTTCTTCC'3	5'GTTGGCGTACAGGTCTTTC'3

The spots chosen had to be consistently expressed or consistently absent on all gels within HaCaT genotype/treatment combination. Selected protein spots were cut from the gel, and the gel plugs containing these spots were preserved in 2% glycerol at 4°C ready for MS analysis. Images of the stained gels were captured with the Biorad 4.2.1 Fluor-S MultiImager system (Biorad) using top illumination fluorescence. Gel image analysis was performed “blind” with Phoretix 2D analytical software (version v2004, Nonlinear Dynamics, Durham, NC). Protein expression was quantified as normalized spot volume, a parameter offered by the Phoretix software which combines spot area and peak height to give an overall expression index and has been used previously in fish proteomics (Smith et al. 2007, 2011).

Mass spectrometry analysis and protein identification. Mass spectrometry analysis was carried out as described by Smith et al. (2007 & 2011) at Queen’s Mass Spectrometry and Proteomics Unit, Ontario, Canada. Approximately 331 protein spot features per sample were detected. Statistical analysis revealed which spots were significantly over- or underexpressed. Eight proteins exhibiting expression changes at any time of the irradiation time course were then pursued for MS and database searches. The selected spots that were cut out from the gel were first treated with ammonium bicarbonate, dehydrated with acetonitrile, and subjected to in-gel trypsin digestion. The digested proteins were concentrated in formic acid, using Millipore C18 ZipTips, and analyzed using a quadrupole time-of-flight (Q-TOF) Global Ultima (Waters, Micromass) with nanoES source; capillary voltage of 1.2 to 1.6 kV and cone voltage of 50 to 100 V. Mass spectra in TOF MS and MS/MS mode were in a mass range of 50 to 1800 m/e, with a resolution of 8000 full width at half maximum height. Argon was used as the collision gas. The MS/MS data were searched using online MASCOT (Matrix Science, United Kingdom) against the National Centre for Biotechnology and Information and the MS protein sequence database. Search criteria were as follows: monoisotopic masses, 1 missed cleavage, tolerances set for 0.3 kDa for peptides matches, and 0.2 kDa for MS/MS fragment matches. All peptide fragments that were obtained for each digest were submitted to online protein database UniProt (UniProt Consortium) for searching.

Real-Time Quantitative PCR

Annexin A2 (*Anxa2*) gene was designed using the online primer design program Primer3, and a list of the forward and reverse sequences for the target gene (*Anxa2*) and housekeeper gene (*Actin*) are displayed in Table 1. *Actin* was chosen as the housekeeper (reference) gene, as it was deemed to be a more reliable endogenous control for the extent of the study

involved, and this was confirmed with careful analysis of raw data. The changes in *Anxa2* expression levels were measured in HaCaT cells grown in ICCM from directly irradiated HaCaT cells (0.05 and 0.5 Gy for 1, 4, 8, and 24 hours), relative to the expression at 0 Gy (control) and normalized to the internal reference gene (*Actin*). The mean-fold changes were calculated using the $\Delta\Delta CT$ mathematical analysis (Livak & Schmittgen, 2001) and mean-fold changes were plotted with GraphPad Prism (GraphPad Software, La Jolla, CA).

MTT assay

The MTT assay measures cell viability (Mosmann, 1983) and was used to measure cell viability in HaCaT cells grown in ITCM derived from the fish explants generated by the ex vivo method described earlier. After the exposure period of 24 hours, both control media (DMEM) and test media (ITCM) were poured off the cells, and the cells were washed with PBS prior to the addition of 100 μ L of fresh DMEM medium (free of FBS and supplements) to each well. The MTT solution (5 mg/mL) was prepared in PBS, and 10 μ L was added to each well and plates were reincubated for 3 hours at 37°C in an atmosphere of 5% CO₂. Following the incubation period, media were discarded, and cells were washed with 100 μ L of PBS and then 100 μ L of dimethyl sulfoxide was added to each well to resolve the formazan crystals and extract the dye. Plates were shaken at 240 rpm for 10 minutes. The reduction of MTT to a blue formazan product was measured at an absorbance of 595 nm on a GENios fluorescence microplate reader. The data (in fluorescence units from the microplate reader) for the test wells were normalized to the assay control (DMEM only), and bystander effects were calculated as a change of viability in the irradiated group compared to the unirradiated group per sample.

Statistical analysis

Normalized spot volumes were expressed as mean values (Figure 1A-D and Figure 2A-D) and compared by applying the statistical 1-way analysis of variance (ANOVA) which tested for the effect of treatment groups followed by post hoc Bonferroni test and corrected for multiple comparisons, with the aid of GraphPad Prism software. A $P < .05$ was considered statistically significant and is marked in figures with $*P < .05$.

Results

Protein Expression

Rainbow trout (*O mykiss*) were exposed to low doses (0.1 and 0.5 Gy) of X-ray radiation in vivo and in vitro explant cultures

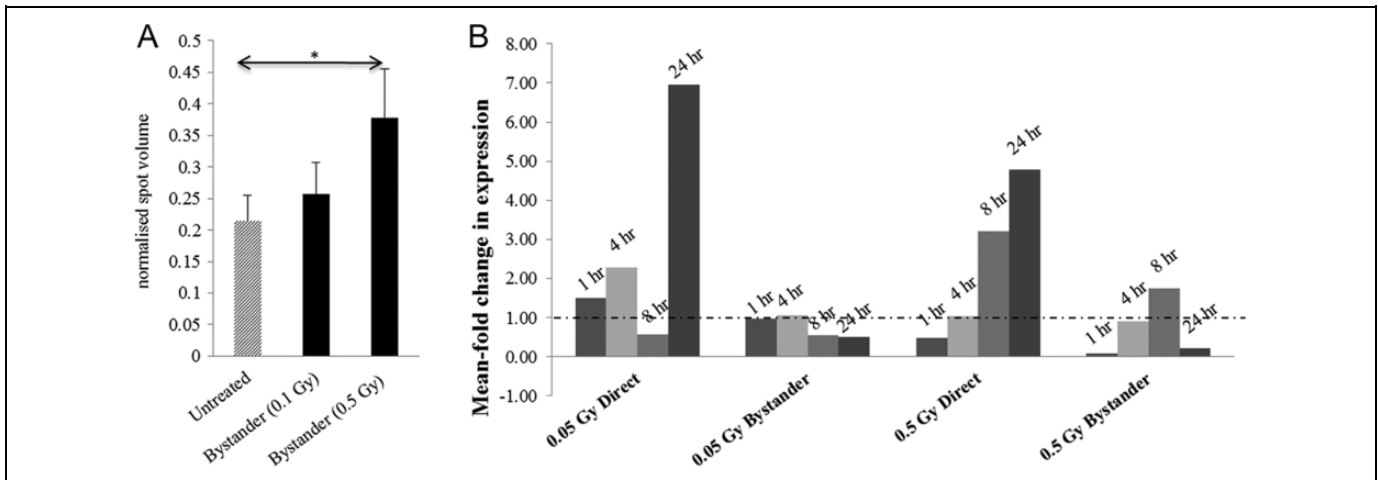


Figure 1. Changes in (A) annexin A2 protein and (B) *Anxa2* gene expression in HaCaT cells following exposure to 0.1 Gy and 0.5 Gy irradiated cell conditioned media (ICCM). Analysis annexin A2 protein and *Anxa2* gene expression were performed on untreated and irradiated tissue conditioned media (ITCM) grown HaCaT homogenates. Data were checked for normality and equal variance, and treatment effects were tested using t-test. Values are expressed as mean \pm standard error of the mean (SEM). * $P < .05$, ** $P < .01$ and *** $P < .001$.

were generated from the skin. The ITCM was harvested from all explant cultures and placed on recipient HaCaT bystander reporter cells. Two-dimensional gel electrophoresis and MS were employed to screen for novel proteins that were significantly over- or underexpressed in the recipient HaCaT cells grown in ITCM. The representative 2D gel is shown in Figure 3 and a list of protein identifications are displayed in Table 2.

HaCaT cells grown in fish ITCM (0.1 and 0.5 Gy) revealed the following changes in protein expression. Annexin A2 was significantly upregulated with exposure to 0.5 Gy ($P = .0161$) and nonsignificantly upregulated 0.1 Gy ITCM (Figure 1A). Cingulin was nonsignificantly upregulated in response to 0.1 Gy and significantly upregulated ($P = .0001$) with 0.5 Gy (Figure 2A). F-actin-capping protein was significantly downregulated ($P = .0366$) in response to both doses of ITCM (Figure 2B). hMMS19 protein was significantly downregulated ($P = .0343$) in response to 0.1 Gy (Figure 2C). EB1 Microtubule-associated protein RP/EB family member 1 (EB1) was significantly downregulated ($P = .0021$) with both doses of ITCM (Figure 2D). Adenomatous polyposis coli 1 (APC 1) was nonsignificantly downregulated ($P = .114$) to exposure of both doses of ITCM (Figure 4A). Rho-GDI2 was significantly downregulated ($P = .0497$) with exposure to 0.1 and 0.5 Gy (Figure 4B). 14-3-3 was significantly downregulated ($p = 0.0269$) with 0.5 Gy exposure (Figure 4C).

Expression of *Anxa2* Gene

The mean-fold changes of *Anxa2* gene expression in HaCaT cells exposed to direct radiation and bystander signals, 0, 0.05, and 0.5 Gy, were quantified at time points of 1, 4, 8, and 24 hours (Figure 1B). The direct 0.05 Gy dose revealed an induction of *Anxa2* expression after 1, 4, and 24 hours. For the 0.05 Gy bystander signals, *Anxa2* was downregulated in expression at all time points. *Anxa2* was upregulated with the direct 0.5 Gy dose

between 8 and 24 hours. On the contrary, the bystander 0.5 Gy dose induced an upregulation at the 8-hour exposure time only.

Cell Viability Assay

The ITCM generated from the fish was then analyzed using the MTT assay, and the data demonstrated that there were individual variation in the RIBE in HaCaT cell cultures receiving this ITCM (Figure 5). The MTT assay illustrated an increase in the surviving fraction in HaCaT cell cultures grown in ITCM (0.1 and 0.5 Gy) in comparison to HaCaT cell cultures exposed to ITCM (0 Gy); however, results were not deemed statistically significant.

Discussion

HaCaT reporter cells were grown in ITCM, previously generated from the skin of X-irradiated fish. The ITCM (0.5 Gy) induced a significant bystander upregulation of annexin A2 and cingulin and a significant downregulation of Rho-GDI2, F-actin-capping protein subunit beta, Microtubule-associated protein RP/EB family member, and 14-3-3 proteins. The ITCM (0.1 Gy) induced a significant bystander downregulation of Rho-GDI2, hMMS19, F-actin-capping protein subunit beta, and Microtubule-associated protein RP/EB family member proteins. A further investigation into of the *Anxa2* transcriptional changes were made to clarify the pattern of expression over time, revealing significant changes in the direct cells in comparison to the bystander cells. And finally, cell viability was measured in HaCaT cells in receipt of 0.1 and 0.5 Gy ITCM harvested from fish explants. The results were suggestive of a proliferative effect in turn triggered by RIBE, however, deemed not statistically significant. This could a well-known cellular response attributable to hormesis, which has been defined as “the stimulation of a system by low doses of substances that are toxic at high doses” (Ryan et al. 2008). The

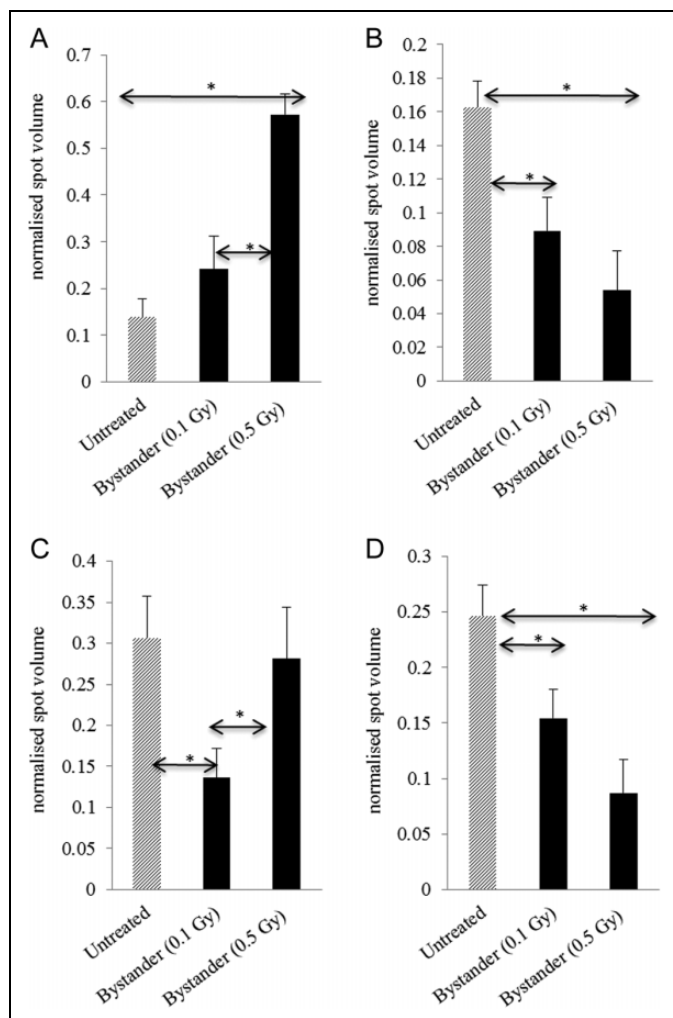


Figure 2. Changes in (A) Cingulin protein, (B) F-actin protein, (C) MMS19 protein, and (D) EB1 protein expression in HaCaT cells following exposure to 0.1 and 0.5 Gy irradiated cell conditioned media (ICCM). Analysis of the proteins were performed on untreated and irradiated tissue conditioned media (ITCM)-grown HaCaT homogenates. Data were checked for normality and equal variance, and treatment effects were tested using *t* test. Values are expressed as mean \pm standard error of the mean (SEM). **P* < .05, ***P* < .01, and ****P* < .001.

aim of the current study was to further investigate the key proteomic changes caused by RIBE to contribute to the understanding of bystander signaling and the risks that may be associated with low-dose exposures. It was of interest to discover whether proteins changing in the direct exposures that were previously investigated by our group were also changed in the reporter HaCaT cells receiving the ITCM.

The current data revealed a significant upregulation of annexin A2 protein. The role of annexins is somewhat contradictory, as there is evidence of expression of the protein in cancers, suggestive of cellular growth (proliferation), and they are associated with cell death (apoptosis). In the current bystander study, we propose that the increased levels of annexin A2 are proportional to reduced levels of apoptosis, as shown by the nonsignificant changes in cellular proliferation, suggestive of

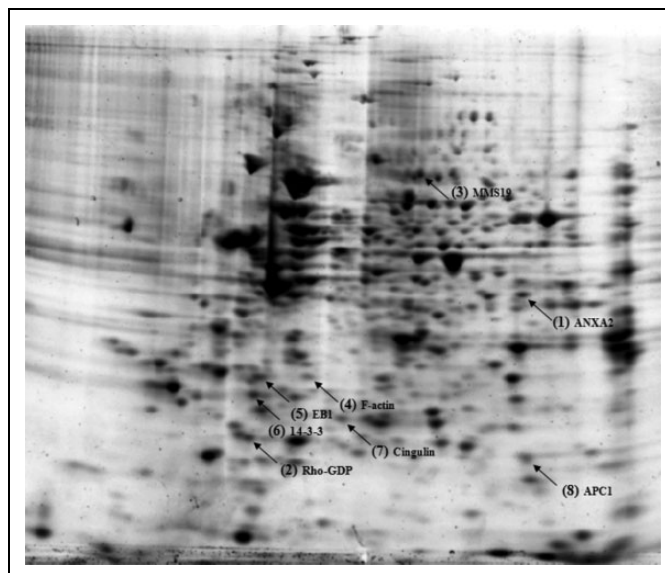


Figure 3. Representative 2-dimensional (2D) gel from HaCaT cells grown in irradiated tissue-conditioned medium from rainbow trout fish skin explants. The proteins selected for 2D gel electrophoresis analysis and their corresponding identifications are marked with arrows on the gel.

cell protection. Whereas direct radiation studies have revealed induced annexin signaling (Smith et al. 2007), which could be indicative of tumorigenesis.

Singh et al. (2007) has shown that annexin A2 is a key regulator of cellular proliferation and found to be highly expressed in gastric cancers in humans. Increased growth may be a protective response supportive of apoptosis as opposed to a tumorigenic response, which would support proliferation. An induction of annexin A2 expression may be suggestive of an immediate protective function and long-term adaption to any consequent radiation exposures. Some studies have recognized annexin A2 as a radioresponsive protein associated with anchorage-independent growth, thus promoting cell growth (Waters et al. 2013). The group showed that annexin A2 may protect cells from radiation-induced apoptosis, by signaling proliferation and potentially leading to tumorigenesis, particularly at low-dose radiation. It has been demonstrated that annexin A2 is secreted into the medium by irradiated cells and can bind to nonirradiated neighboring cells in the vicinity, inducing anchorage-independent growth (Weber et al. 2005 & 2009). Perhaps Annexin is playing a role in protection.

Oppositely, annexins have been described to have roles as effectors, regulators, and mediators of Ca^{2+} signals, a known regulator of apoptosis (Gerke and Moss, 2002), so it is possible that the function of annexin A2 is dependent on the source of damage and the cell type. Annexin A2 has been shown to contribute to radiation-dependent regulation of transcription and cell fate, whereby “silencing” A2 can lead to an increase in cell death, perhaps suggesting a possible role for protection of the cell from damage such as radiation (Waters et al. 2013). The study revealed that cells depleted levels of annexin A2 induced more oxidative DNA damage than control cells, in

Table 2. Peptide Ion Identification Information for Proteins Indicated in Spot Volumes.

Spot Number	Protein Description	Database/ Accession No.	Peptide Sequence	Analysis
168	Annexin II	PO4272	136-TNQELQEINR-145	MS/MS
299	Rho-GDP	P52566	51-TLLGDGPVVTDPK-63	MS/MS
53	MM519 nucleotide excision repair protein homolog	E7FBU4	380-ASIGtQAVIPALLDQYBBR-399	MS/MS
254	F-actin capping protein subunit beta	Q5R507	96-LEVEANNAFDQYR-108	MS/MS
256	Microtubule-associated protein RP/EB family member I	Q15691	183-NPGVGNNGDDEAAELMQQVNVLK-204	MS/MS
269	14-3-3 protein beta/alpha	P31946	141-QTTVSNSQQAYQEAFEISK-159	MS/MS
290	Cingulin	B1MTG4	653-DRELEKQLAGLR-664	MS/MS
313	Anaphase-promoting complex subunit I	P53995	544-LLGSMDEVLLSPVPELRDSsK-565	MS/MS

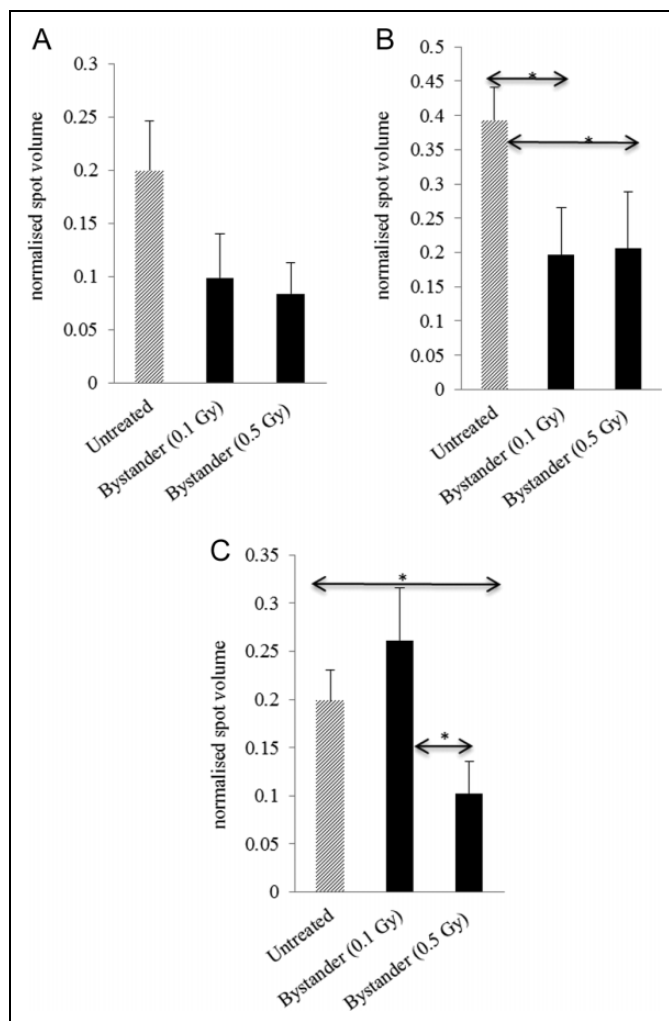


Figure 4. Changes in (A) APC protein, (B) RHO-GDI2 protein, and (C) 14-3-3 protein expression in HaCaT cells following exposure to 0.1 and 0.5 Gy irradiated cell conditioned media (ICCM). Analysis of the proteins were performed on untreated and irradiated tissue conditioned media (ITCM)-grown HaCaT homogenates. Data were checked for normality and equal variance, and treatment effects were tested using *t* test. Values are expressed as mean \pm standard error of the mean (SEM). **P* < .05, ***P* < .01, and ****P* < .001.

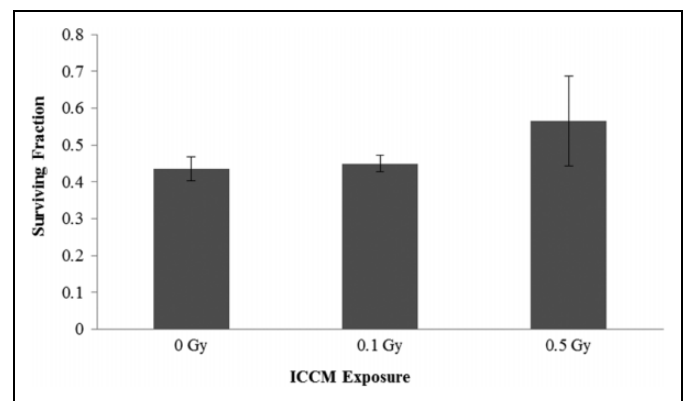


Figure 5. The surviving fractions of HaCaT cells grown in irradiated tissue conditioned media (ITCM) harvested from irradiated fish explants (0, 0.1, and 0.5 Gy).

response to IR. Annexin A2 accumulates in the nucleus in response to DNA-damaging agents (X-ray), suggesting that annexin A2 may play a role in protecting DNA from oxidation by ROS. Annexin A2 can bind RNA impacting on RNA stability and subsequent protein expression in cells, which may influence on the overall fate of the cells (mRNA stability; Filipenko et al. 2004).

In the current study, the annexin A2 gene (*ANAX2*) was investigated for changes in expression in HaCaT cells direct and bystander exposed to 0.05 and 0.5 Gy IR for 1, 4, 8 and 24 hours. The 0.05 Gy dose was chosen to gain additional knowledge of the unique low-dose bystander responses. Exposure to ICCM (0.05 Gy) revealed no significant change in expression, and exposure to ICCM (0.5 Gy) irradiation instigated an increase in expression of the gene after 8 hours and a significant reduction in expression of the gene after 1 and 24 hours. A previous study by our group consolidated the role of apoptosis in both a direct and a bystander response, revealing that bystander irradiation initiated a modulated apoptotic response in HaCaT cells grown in ICCM (0.05 and 0.5 Gy; Furlong, Mothersill, Lyng, & Howe, 2013). The data suggested that apoptosis was initiated and not fully executed by the executioner caspases, possibly due to intervention of other cell death

pathways. In our current investigation, HaCaT cells had increased levels of annexin A2 and as previously mentioned may have an apoptotic role, particularly as there was no statistical change in cell proliferation, thus suggesting increased protection of the cells tumorigenesis.

14-3-3 protein was significantly downregulated in HaCaT cells after exposure to ITCM from fish explants that received 0.5 Gy only. However, the pattern of expression was similar to a hormetic inverted U-shaped dose–response curve, indicative of a low-dose stimulation (Cook and Calabrese, 2006). Some of the 14-3-3 binding antagonizes the proapoptotic activity of Bad (Bcl-2 family) and competes with proapoptotic proteins important for cell death signaling processes. A loss of 14-3-3 expression disturbs the multiple regulatory pathways and is an early event in neoplastic transformation, carcinogenesis, and subsequently increasing radiosensitivity (Nakajima et al. 2003; Mhawech, 2005). Decreased levels of the protein could be a positive marker in sensitizing human cancers to radiation. There may be an integrated function of the 14-3-3 and annexin A2 in terms of apoptosis and how this may relate to tumor protection.

Expression of cingulin protein was significantly upregulated in HaCaT cells after exposure to ITCM from fish explants that received 0.5 Gy only. Cingulin is known to be important for the formation and regulation of tight junctions (TJs) in cells and is located at the surfaces of TJs (He et al. 2007). Cingulin is usually recruited to cell–cell junctions and responsible for gene expression regulation, cell proliferation, and cell density working in conjunction with RhoA activator GDP/GTP-exchange factor signaling pathway (Guillemot and Citi, 2006). In vitro studies have shown that cingulin interacts with various components of TJs including F-actin suggestive of a role for cingulin as a linker between the TJ membrane and F-actin cytoskeleton reorganization (Bazzoni et al. 2000; Ohnishi et al. 2004). F-actin was identified in this study as it was significantly downregulated in HaCaT cells receiving ITCM from fish. The cytoskeletal protein is important for cytoskeletal and cell morphology organization (Maruyama, et al. 1990). Increased expression of cingulin and decreased levels of F-actin may be reflective of exposure to damage and induction of a reparative mechanism.

Expression of Rho-GDP dissociation inhibitor (GDI) 2 protein was significantly decreased in HaCaT cells after exposure to ITCM from fish explants that received 0.1 and 0.5 Gy. The Rho-GDI's are regulators of Rho-GTPase, which are associated with regulation of actin dynamics, gene transcription, and motility (Bishop and Hall, 2000). Rho-GDI's escort GTPases to specific membrane signaling complexes, protecting them from degradation (Zhang, 2006). The role of RhoGDI as a regulator of epithelial apical/basolateral polarity via the regulation of GTPase activity is well established (Fukata et al. 2003). The GDIs are central to the fundamental processes of intercellular signaling and transport (Seabra and Wasmeier, 2004). Deregulated RhoGDI2 expression has been found in various cancers (DerMardirossian and Bokoch, 2005; Dovas and Couchman, 2005; Ellenbroek and Collard, 2007), and it is thought that the reduced levels of the protein is linked to deregulated transmission of growth signaling. The decrease in expression of Rho-

GDI observed in the current study could be indicative of decreased proliferation. The current study also contrasts with a previous study in which Rho-GDI was increased in bystander fish (Smith et al. 2007). It is clear from the current data that the signal is not inducing the same response in HaCaT cells as it does in fish. The RHO-GDI is a regulator of polarity, which is essential for the freshwater fish gill. However it is also associated with cancer. The present study revealed an opposing effect of RHO-GDI found previously (Smith et al. 2007). One explanation for this is that the current study investigated a fish-to-HaCaT cell bystander effect as opposed to a fish-to-fish effect discussed by Smith et al. (2007). It is possible that HaCaT cells may not require polarity as much as a gill does in vivo. In summary, reduced expression of RHO-GDI in the current study could be indicative that the HaCaT cells are avoiding the development of cancer (tumorigenesis), and this mechanism is taking priority over polarity, whereas in fish exposures, polarity was a more urgent concern.

The hMMS19 protein was significantly downregulated in HaCaT cells after exposure to ITCM from fish explants that received 0.1 Gy only. Although the changes with the 0.5 Gy were not significant, it is noted that the response revealed a U-shaped dose–response curve. The changes are similar to the hormetic dose–response curve which describes a low-dose reduction and high-dose development of harmful effects in response to irradiation (Ryan et al. 2008) and has been discussed in previous radiation investigations (Cook and Calabrese, 2006). The protein may be attempting to repair damage signaled from the bystander ITCM. Microtubule-associated protein RP/EB1 was significantly downregulated with both doses. The EB1 is capable of binding to the end of microtubules to regulate the dynamics of the microtubule cytoskeleton, specifically promoting cytoplasmic microtubule nucleation and elongation. Their role in spindle function is thought to occur through stabilization of microtubules, anchoring them at centrosomes (Askham et al. 2002; Hayashi et al. 2005; Honnappa et al. 2009; van der Vaart et al. 2011). The reduced levels of EB1 perhaps cause dysregulation of the actin cytoskeletal network. The EB1 can interact with the tumor suppressor APC protein which plays a role in the movement of chromosomes to opposite poles of the cell during cell division (Jin et al. 2008); however, there were nonsignificant decreased levels of APC1.

The direct effects of radiation in fish are well established and documented and for that reason were not included in this study (Mothersill et al. 2012, 2011, 2010b, 2006; Smith et al. 2007). Experiments measuring cell survival, using reporter cells receiving ITCM from irradiated rainbow trout (*Onchorhynchus mykiss*; O'Dowd et al. 2006), zebrafish (*D rerio*; Mothersill et al. 2007) and medaka (*O latipes*; Mothersill et al. 2009) have all been shown to produce direct and bystander effects. In addition, the proteomic changes have been described in rainbow trout (*O mykiss*) gills (Mothersill et al. 2006). Due to the complexity of tissues in comparison to cell cultures, it is believed that fish may be capable of producing and “organizing” the initial bystander signal, whereas HaCaT cell cultures in the current study have a separate unique response.

The current study was an exploratory investigation to determine whether there are proteins commonly expressed in direct and bystander responses utilizing the HaCaT reporter cell line. Significant changes in protein expression levels were revealed and unique between the 2 doses of ITCM from fish. The data revealed changes in proteins that may influence apoptosis, an induction of cell communication, and reparative signaling along with deregulation of transmission of growth signals. It was hypothesized that a “protective” mechanism may be in place in HaCaT cells responding to oxidative stress generated in the cells grown in fish ITCM. This strongly determines the need for more meticulous risk assessments of low-dose irradiation exposures, particularly with regard to human exposure and considering the potential long-term effects.

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Authors' Note

Hayley Furlong and Richard Smith carried out all experimental procedures with contribution from Jiayi Wang. Colin Seymour, Carmel Mothersill and Orla Howe were responsible for supervision and development of the project.

Declaration of Conflicting Interests

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